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(54) Title: DUAL-EFFECT LIGANDS COMPRISING ANTI-INFLAMMATORY HSP PEPTIDE EPTOPES FOR IMMUNOMODULATION

(57) Abstract: The present invention discloses anti-inflammatory Hsp60 derived peptides including a minimal epitope of the known Peptide p277 that are capable of reacting via the Toll like receptor 2 (Tlr2) on T cells, without necessarily activating the TCR of these cells. This minimal epitope serves as a basis for the design of novel dual effect ligands, having as a first part a Tlr2 epitope and as a second segment a specific peptide capable of eliciting a reaction via a T cell receptor. The specific peptide of the second segment is typically an antigenic epitope that is specific for a cell mediated autoimmune disease. This antigenic fragment will provide specificity via the TCR, while the Tlr2 specific epitope derived from Peptide p277 will provide the means of diminishing damaging immune responses and enhancing beneficial immune responses. The anti-inflammatory peptides and dual effect ligands of the invention will be useful in treating autoimmune diseases, inflammatory conditions and graft rejection.

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DUAL-EFFECT LIGANDS COMPRISING ANTI-INFLAMMATORY HSP PEPTIDE EPITOPES FOR IMMUNOMODULATION

Field of the Invention

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The present invention provides novel conjugates comprising Hsp60-peptides and their uses in treating immune conditions particularly inflammatory conditions and autoimmune diseases. The present invention particularly relates to the anti-inflammatory effects of Hsp60 derived peptide conjugates including the known Hsp60
10 peptide p277 via its innate receptor and provides novel uses for p277 and its derivatives in treating immune conditions and autoimmune diseases.

Background of the Invention

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Members of the 60 kDa heat shock protein (HSP60) family of molecules are recognized both by the antigen receptors of the adaptive T and B lymphocytes and by innate immune receptors of endothelial cells, smooth muscle cells, and macrophages. (1). As an antigen, HSP60 functions as a dominant immunogen both in foreign immunity and in autoimmunity. Bacterial HSP60, for example, has been identified as
20 the "common bacterial antigen" recognized by T cells and antibodies in natural infection and vaccination. Autoimmunity to HSP60 was first noted in adjuvant arthritis (2), and then demonstrated in Type I diabetes in mice (3) and later in humans (4). Moreover, HSP60 autoimmunity has been associated with inflammatory conditions such as atherosclerosis, Behçet's disease, lupus, and uveitis.

25

HSP60 has also been discovered to function as a ligand for an innate immune receptor (5), and this may account for the strong immunogenicity of HSP60. HSP60 can activate macrophages by way of the innate toll-like receptor 4 (TLR-4) to induce enhanced production of NO and IL-12, IL-15, TNF α , IL-6 and other Th1-type
30 cytokines (5). Thus, the power of HSP60 as an immunogen or autoimmunogen might be explained by its combined signaling of lymphocytes along with activation of antigen presenting cells (APC). Thus, HSP60 has been found to signal different types of immune cells using different classes of receptors: T cells by way of adaptive antigen receptors, and macrophages by way of an innate receptor TLR-4.

Peptide p277(Val⁶Val¹¹), also known as DiaPep277, disclosed in US 6,180,103, is a synthetic analog of a native 24-amino acid fragment p277 of the 60kDa human HSP60. Previous work has revealed that Peptide 277 is a target for
5 spontaneous autoimmune T cells in Type I diabetes in both NOD mice (6) and human diabetes patients (4). Therapeutic vaccination using p277 can arrest the progression of β -cell destruction in NOD mice and in human subjects with new onset Type I diabetes (7). Preservation of β cells by p277 vaccination is associated with the activation of a Th2 response to p277 and by down-regulation of other autoimmune reactions
10 associated with Type I diabetes. Vaccination with p277 safely leaves intact immune reactions to other antigens not involved in the autoimmune diabetes process (7).

Numerous disclosures claim uses of heat shock proteins or fragments thereof as immune modulators in diagnosis, treatment or prevention of autoimmune diseases.
15 Many of these disclosures relate to the human HSP60 or to the bacterial equivalent hsp65, or fragments thereof.

For example, the particular protein produced by the human body during development of Insulin dependent diabetes mellitus (IDDM), which serves as a
20 diagnostic marker for the incipient outbreak of IDDM, is the human heat shock protein having a size of about 62 kD (human HSP60) or an antigen cross-reactive therewith as disclosed in EP 0417271, and in US Patents 5,114,844; 5,578,303 5,671,848; and 5,780,034. It has been disclosed that fragments of this hsp60 protein may serve as therapeutically useful entities in preventing or alleviating IDDM (US
25 Patent 6,180,103, WO 96/19236, and WO 97/01959).

Hsp60 and fragments or synthetic analogs of Hsp60 including p277 have been disclosed as being effective in decreasing the severity of host-versus-graft disease (US Patent 5,993,803 and WO 98/08536).

In addition, fragments of hsp60 may be used as carriers for development of
30 synthetic vaccines by increasing the immunogenicity of poorly immunogenic antigens as disclosed in US Patents 5,736,146 and 5,869,058.

European Patent 0262710 discloses polypeptides useful for alleviation, treatment, and diagnosis of autoimmune arthritis and similar autoimmune diseases.

The claimed polypeptides are derived from bacterial protein named "Antigen A" which was identified later as Mycobacterial hsp65.

WO 92/04049 discloses peptides of at least seven amino acids homologous to a fragment of Mycobacterium tuberculosis hsp65, which inhibit T-lymphocytes activation and proliferation and can protect from immune reactions and immune-related disease.

WO 89/12455 and WO 94/29459, disclose the use of stress proteins and analogs for producing or enhancing an immune response or for inducing immune tolerance, for prophylaxis or therapy of autoimmune diseases and for treating or preventing infections or cancers. A fusion protein is claimed comprising a stress protein fused to a protein against which an immune response is desired.

WO 95/25744 discloses microbial stress protein fragments containing epitopes homologous to related mammalian epitopes – used to treat and prevent inflammatory autoimmune diseases and to prevent transplant rejection. The protective epitopes are located in short peptides comprising 5-15 amino acid sequences regions of stress proteins, that are highly conserved between microorganisms and animals.

WO 97/11966 and WO 96/10039 disclose polypeptides of up to 21 amino acids, derived from microbial heat shock protein which are useful for prophylaxis or treatment of autoimmune diseases especially arthritis.

WO 96/16083 discloses a peptide 25 amino acids long, derived from the 10 kD heat shock protein (hsp10) of Mycobacterium tuberculosis which is useful in pharmaceutical products for the treatment of inflammatory pathologies, especially rheumatoid arthritis.

WO 91/02542 discloses the use of antigenic and/or immuno-regulatory material derived from Mycobacterium vaccae and specifically hsp60, for treating chronic inflammatory disorders caused or accompanied by an abnormally high release of IL-6 and/or TNF- α .

WO 96/18646 discloses peptides of 9-20 amino acids derived from Mycobacterial hsp60 used for treatment or prevention of autoimmune CNS diseases, e.g. multiple sclerosis, chronic inflammatory CNS disease and primary brain tumors.

WO 94/02509 discloses peptides of 7-30 amino acids derived from DR3-restricted epitope of Mycobacterial hsp60 used for treatment of HLA-DR3 related autoimmune diseases.

WO 00/27870 discloses peptides derived from Mycobacterial and rat hsp60 and vaccines comprising such peptides for immunization against autoimmune and inflammatory diseases.

5 US 5,958,416 describes heat shock protein peptides and methods for modulating autoimmune central nervous system diseases.

WO 01/43691 discloses a variant, derivative, analog or peptide fragment of hsp60, the variant, derivative, analog, or peptide fragment having the ability to act as an antagonist of hsp60 characterized by its ability to reduce or prevent the induction of a pro-inflammatory immune response of cells of the innate
10 immune system by hsp60.

T cells involved in immune responses have been shown to be modulated by Hsp60 and undergo a shift between helper cell types Th1 and Th2, that is beneficial to halting the progression of autoimmune diseases and in attenuating Hsp60 related autoimmunity. T cells move to sites of inflammation by way of response of their
15 chemokine receptors. Following their passage through the blood-vessel endothelium, migrating T cells must interact with components of the extracellular matrix (ECM), particularly with glycoproteins such as collagen, laminin and fibronectin (FN). The β 1-integrin molecules of T cells enable them to adhere to and migrate through the ECM, a process which is affected by a variety of cytokines, chemokines, and acute
20 phase proteins situated within the context of the ECM. Unlike antigen recognition, the physiology of T-cell migration and adhesion is accomplished without the mediation of APC.

There exists a long-felt need for an effective means of curing or ameliorating inflammatory conditions and autoimmune diseases. None of the background art
25 discloses HSP60-peptide dual effect conjugates effective in treating specific inflammatory conditions and autoimmune diseases.

Summary of the Invention

The present invention identifies the innate receptor relevant to therapeutic
30 utility of the HSP60 derived peptide p277, and thereby enables the design and characterization of novel derivatives of p277, useful for treating additional autoimmune diseases and conditions amenable to intervention. The present invention

further provides novel uses for p277 and its derivatives in treating immune conditions including but not limited to autoimmune diseases in addition to the known uses for treatment of IDDM and graft rejection. The terms p277 and Peptide p277 are used interchangeably throughout the specification and in the claims and these terms are
 5 intended to denote both the native sequence (SEQ ID NO:1) as well as synthetic variants thereof, such as DiaPep277 (SEQ ID NO:2).

The present invention is based on part on the unexpected discovery that certain peptide epitopes derived from hsp60 do not exert their effect as heretofore believed by acting either at the level of the Toll-like receptor 4, nor do they act at the level of
 10 antigen presenting cells.

The present inventors have now made the unexpected discovery that HSP60 or peptide p277 can activate T cells, both naïve and memory, through an innate pathway without the mediation of antigen presenting cells. This activation depends on the Toll-like receptor 2 (TLR2) molecule, and not on the TLR4 molecule associated with
 15 macrophage activation. The T cells respond to very low concentrations of p277 relative to the concentrations of whole HSP60 required to activate macrophages. The important feature of this p277 innate effect on T cells is that the activation is anti-inflammatory.

According to one aspect, the present invention provides compositions and
 20 methods for treating inflammatory conditions and diseases by inhibiting T cell chemotaxis towards chemokines. The compositions comprise heat shock protein 60 (Hsp60), or an active fragment thereof, which act via T cell innate receptor Toll like receptor 2 (TLR-2). In one embodiment, the active fragment of HSP60 is the DiaPep277 (p277). In another embodiment, said fragment is a minimal epitope of
 25 p277, that retains the ability to elicit its action via the Tlr-2.

According to another aspect it is now disclosed that Peptide p277 acts as a signal that activates T-cell adhesion to the extra-cellular matrix protein fibronectin (FN) and inhibits T-cell chemotaxis towards chemokines that signal T cells to migrate to inflammatory sites. Moreover, p277 down-regulates the expression of chemokine
 30 receptors such as CXCR4 and CCR7. These new findings provide ways to optimize the anti-inflammatory effects of p277 via its innate receptor and indicate novel uses for p277 and its derivatives in treating immune conditions in autoimmune disease besides the known uses in IDDM and graft rejection.

According to another aspect of the present invention it is possible to characterize and isolate a minimal epitope of DiaPep277. The minimal epitope of p277 will be an epitope that is still capable of eliciting its action via the Tlr2 receptor on the T cells, without necessarily activating the TCR of these cells.

- 5 According to another aspect the present invention provides methods for the use of p277 or its minimal epitope to treat conditions dependent on chemokine receptors. These conditions include diseases where one or more chemokine receptor may serve as the receptor for a lethal virus, as exemplified by HIV infection.

- This minimal epitope can serve as a basis for the design of novel Dual Effect
10 Ligands, having as a first part a peptide epitope capable of eliciting a reaction via Tlr2 and as a second segment a specific peptide capable of eliciting a reaction via a T cell receptor.

- The specific peptide of the second segment is typically an antigenic epitope that is specific for a cell mediated autoimmune disease. This antigenic fragment will
15 provide specificity via the TCR, while the Tlr2 specific epitope derived from Peptide p277 will provides the means of diminishing damaging immune responses and enhancing beneficial immune responses.

- In one embodiment of the present invention, the epitope of HSP60 or p277 exerting its effect via TLR-2 binding is conjugated to a second segment capable of
20 eliciting a reaction via a T cell receptor (TCR). The peptides can be conjugated directly via an amide bond, synthesized as a dual ligand peptide, or joined by means of a linker moiety as is well known in the art to which the present invention pertains.

- The second segment capable of eliciting a reaction via a TCR as used in the present invention, can be an antigen involved in any one or more of the following
25 autoimmune diseases or disorders: diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, myasthenia gravis, systemic lupus erythematosus (SLE), autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjogren's Syndrome, including keratoconjunctivitis sicca secondary to
30 Sjogren's Syndrome, alopecia areata, allergic responses due to arthropod bite reactions, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing

hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Graves ophthalmopathy, sarcoidosis, primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis.

Examples of known antigens involved in autoimmune diseases include but are not limited to myelin basic protein, myelin oligodendrocyte glycoprotein and myelin proteolipid protein (involved in multiple sclerosis), acetylcholine receptor components (involved in myasthenia gravis), collagen and Mycobacterial hsp peptide 180-188 (involved in arthritis), laminin and p53 peptide (involved in systemic lupus erythematosus). Certain exemplary peptides of these known antigens useful in the compositions and methods of the present invention are denoted as SEQ ID NOS:3-11 hereinbelow.

It is to be understood that the compositions and methods of the present invention are amenable for use with these and other known peptide antigens that have been implicated as T cell epitopes involved in autoimmunity. Clearly the present invention is intended to encompass any other such peptide antigen that may in future be disclosed that may be used as the TCR interacting segment of the Dual Effect Ligands according to the principles of the present invention.

All these novel conjugated peptide compounds are also useful for diagnosis or monitoring the progression of these diseases.

The present invention further relates to pharmaceutical compositions comprising a peptide analog according to the invention. The formulation of said compound into a pharmaceutical composition further comprises the addition of a pharmaceutically acceptable carrier, excipient and/or diluent. These pharmaceutical compositions according to the present invention comprise at least one peptide analog of a heat shock protein.

These pharmaceutical compositions may be administered by any suitable route of administration, including orally, topically, transdermally or systemically. Preferred modes of administration include but are not limited to parenteral routes such as subcutaneous, intravenous and intramuscular injections. For the pharmaceutical compositions comprising peptides, parenteral routes of administration are generally required. However in certain embodiments, additional preferred routes of administration include but are not limited to administration via nasal inhalation or oral

ingestion. For oral ingestion it is possible to prepare peptide analogs or specific peptide formulations having improved oral bioavailability and enhanced resistance to degradation as are known in the art.

According to additional aspects of the invention methods are provided of using
5 the novel Dual Effect Ligands for diagnosis, treatment and prevention of diseases and disorders that are amenable to immune intervention.

The present invention also provides a method of treating an inflammatory condition or disorder, or a disorder dependent on chemokine receptors, comprising administering to a patient in need thereof a pharmaceutical composition comprising
10 cells exposed ex vivo to an anti-inflammatory effective amount of HSP60 or an active fragment thereof. In one embodiment, the active fragment of HSP60 is the DiaPep277 (p277). In another embodiment, said fragment is an active fragment of p277.

The present invention also provides a method of inhibiting the migration of T cells comprising exposing the T cells to soluble HSP60 or an active fragment thereof.
15 In one embodiment, the active fragment of HSP60 is the DiaPep277 (p277). In another embodiment, said fragment is an active fragment of p277. The exposure of T cells to HSP60, causes adherence of the T cells to a substrate comprising extracellular matrix components, such as fibronectin, thereby inhibiting the migration of said T cells.

The present invention also provides a method of inhibiting the release of IFN γ from activated T cells comprising exposing the T cells to soluble HSP60 or an active fragment thereof. In one embodiment, the active fragment of HSP60 is the DiaPep277
20 (p277). In another embodiment, said fragment is an active fragment of p277.

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Brief Description of the Figures

Figure 1 demonstrates that treatment with p277 activates T-cell adhesion to FN. Concentrations of p277 as low as 0.01 ng/ml (for CD45RA+ naïve T cells) or 1 μ g/ml
30 (for CD45RO+ memory T cells) can enhance adhesion to FN.

Figure 2 demonstrates that the pro-adhesive effect of p277 depends on β 1 integrins. The pro-adhesive effect of p277 on T cells could be inhibited by antibodies to VLA-4 or VLA-5, but not by an antibody to VLA-6.

Figure 3 demonstrates that TLR2, and not TLR4, is required for the pro-adhesive effect of p277 on T cells. A monoclonal antibody to TLR2 significantly inhibited p277-induced adhesion; anti-TLR4 had no effect.

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Figure 4 demonstrates that treatment with p277 blocks T-cell migration to a chemokine gradient. p277 treatment can block the migration of both naïve (CD45RA+) and memory (CD45RO+) T cells towards the chemokine SDF-1 α .

- 10 Figure 5 demonstrates that treatment with p277 inhibits the expression of chemokine receptors on T cells. The expression of both CXCR4 and CCR7 were inhibited by treatment of either naïve (CD45RA+) or memory (CD45RO+) T cells.

- 15 Figure 6 demonstrates that signal transduction via protein kinase C (PKC) is required for the effect of p277 on chemokine expression. p277 inhibition of CXCR4 expression was blocked by the PKC inhibitor GF10923.

- 20 Figure 7 demonstrates that treatment with p277 in vivo can inhibit a delayed hypersensitivity reaction. The intravenous injection of 0.01 ng given twice can inhibit by 40% a delayed hypersensitivity reaction elicited to the skin sensitizer Oxazolone. Treatment with an injection of 200 μ g of dexamethasone (the gold standard anti-inflammatory drug) inhibited the reaction by 75%.

- 25 Figure 8 shows the effect of p277 delivered by constant infusion of 100 ng per hour over 7 days. Here, p277 inhibited the reaction by 30%; dexamethasone treatment inhibited the reaction by 35%.

- 30 Figure 9 demonstrates that HSP60 induces CD45RA⁺ and CD45RO⁺ human T cell adhesion to FN. Analysis of resting or IL-2-activated naïve and memory T cell adhesion to FN.

Figure 10 demonstrates the GroEl and LPS induction of CD45RA⁺ and CD45RO⁺ T-cell adhesion to FN.

Figure 11 demonstrates the signaling mechanisms involved in HSP60-induced CD45RA⁺ T cell adhesion.

- 5 Figure 12 demonstrates that toll like receptor 2 (TLR-2), but not toll like receptor 4 (TLR-4) is involved in HSP60-induced T cell adhesion.

Figure 13 demonstrates that HSP60 modulates CD45RA⁺ T-cell chemotaxis to SDF-1 α (A) and ELC (B), and actin polymerization (C) induced by SDF-1 α .

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Figure 14 demonstrates that HSP60 down-regulates CXCR4 (A) and CCR7 (B) expression on human T cells in a TLR-2 and PKC-dependent manner(C).

- 15 Figure 15 demonstrates that HSP60-treatment (18 hr) inhibits CD45RA⁺ and CD45RO⁺ T-cell chemotaxis to SDF-1 α .

Figure 16 demonstrates that HSP60 treatment (18 hr) inhibits mouse lymph node cell-chemotaxis to SDF-1 α (A) and adoptive transfer DTH (B).

- 20 Figure 17 demonstrates the in vivo inhibition of delayed type hypersensitivity (DTH) with HSP60.

Figure 18 shows that HSP60 inhibits IFN- γ secretion by T cells activated by mitogenic anti-CD3.

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Figure 19 shows that HSP60 inhibits IFN- γ secretion by T cells activated by chemokine SDF-1 α and mitogenic anti-CD3.

- 30 Figure 20 shows that HSP60 inhibits IFN- γ secretion by T cells activated by chemokine RANTES and mitogenic anti-CD3.

Figure 21 shows that HSP60 inhibits IFN- γ secretion by T cells activated by cytokine IL-2 and mitogenic anti-CD3.

Detailed Description of the Invention

The present invention provides novel uses for HSP60 or an active fragment thereof in treating autoimmune diseases, chronic inflammatory disorders, acute inflammatory disorders, disorders dependent on chemokine receptors and graft rejection. In one embodiment, the active fragment of HSP60 is a fragment of the peptide denoted p277. The native amino acid sequence of p277 fragment comprises 24 amino acids as disclosed in US 5,671,848. The amino acid sequence of p277 is denoted herein as SEQ ID No. 1: Val Leu Gly Gly Gly Cys Ala Leu Leu Arg Cys Ile Pro Ala Leu Asp Ser Leu Thr Pro Ala Asn Glu Asp. In another embodiment, said fragment is an epitope of a variant of p277 as disclosed in US 6,180,103. The amino acid sequence of this variant is denoted herein as SEQ ID No. 2: Val Leu Gly Gly Gly Val Ala Leu Leu Arg Val Ile Pro Ala Leu Asp Ser Leu Thr Pro Ala Asn Glu Asp (DiaPep 277 Val⁶Val¹¹).

The present inventors have now made the unexpected discovery that HSP60, or an active fragment thereof can inhibit T cell chemotaxis towards chemokines, through an innate pathway without the mediation of antigen presenting cells. In one embodiment, the active fragment is p277, or a minimal fragment thereof. The inhibition of T cell migration towards chemokines depends on the activation of TLR-2 molecule located on T cells by HSP60, or the active fragment thereof. The T cells respond to very low concentrations of HSP60 or p277 relative to the concentrations of whole HSP60 required to activate macrophages. The important feature of this HSP60 or p277 innate effect on T cells is that the activation is anti-inflammatory.

It is now disclosed that HSP60 or p277 acts as a signal that activates T-cell adhesion to the extra-cellular matrix protein fibronectin (FN) and inhibits T-cell chemotaxis towards chemokines that signal T cells to migrate to inflammatory sites. Moreover, HSP60 or p277 down-regulates the expression in T cells of chemokine receptors such as CXCR4 and CCR7. The chemokine receptor CCR7 is required for T cell migration to the Th1 areas of lymph nodes, and this effect of HSP60 or p277 could explain the enhancement of anti-inflammatory Th2 reactivity in place of pro-inflammatory Th1 activity.

Moreover, it is now disclosed that HSP60 inhibits IFN- γ secretion by T cells activated by various pro-inflammatory factors, such as mitogenic anti-CD3, chemokine SDF-1 α , chemokine RANTES and by cytokine IL-2. Therefore, it is now disclosed for the first time that innate activation of T cells by HSP60 (via the TLR-2

pathway) could down-regulate the production of IFN- γ . The present invention provides novel uses for HSP60 or p277 and its derivatives in treating immune conditions in autoimmune disease and graft rejection.

The present discoveries of the innate actions of p277 provide a mechanism to explain the anti-inflammatory effects of p277 in type 1 diabetes. It is now disclosed that p277 works on T-cell immunity as a dual-effect ligand (DEL): p277 is both an antigen and an innate regulatory signal. Peptide p277 is an antigen for T-cell clones involved in the autoimmune response, but, as we now show, it is also an innate ligand for a TLR2-dependent pathway in T cells. Peptide p277 is a DEL: p277 as an antigen provides the specificity of the response to diabetes-associated autoimmunity, and as a ligand for an anti-inflammatory innate receptor, p277 changes the phenotype of the autoimmunity from a Th1 to a Th2 reaction. Such is the power of the DEL.

Novel applications for p277 in immunomodulation.

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In the light of the unexpected discoveries described here, the following preferred embodiments will become clear to any person familiar with the art:

1. Definition of minimal p277 epitope for activating the T-cell innate receptor TLR2 pathway. The p277 sequence can be shortened by deleting amino acid residues from each end of the molecule until the smallest effective p277 derivative is obtained. Adhesion to FN or inhibition of chemokine receptors are very convenient assay systems to detect the peptide derivatives that activate the innate T-cell response.
2. Optimization of p277 derivatives. Once the minimal effective innate sequence of p277 is identified, it would be quite easy to substitute each position with Alanine and uncover the key residues required for binding to the innate T-cell receptor. These key residues can then be substituted to discover more effective variant peptides. Likewise, the same amino acid substitution procedure can be used to discover inhibitor peptides: blocking peptides that bind, but do not activate the anti-inflammatory effect.
3. Non-peptide analogues. Once the minimal and optimal peptide is characterized, it would be possible to synthesize non-peptidic analogues that would have the structure of p277 variants, but which would be resistant to

enzymatic degradation: see for example ref 8. Such analogues would have extended half-lives and be more effective in their anti-inflammatory effects.

4. Constructing novel and specific DEL immunomodulators for additional immune indications. The p277 variant most effective in innate anti-inflammatory T-cell activation could be combined with molecules or peptides known to be targeted as antigens in other autoimmune diseases to create effective DEL immunomodulators. For example, the p277 variant plus a myelin antigen or peptide would be an effective treatment for multiple sclerosis (9); p277 variant plus acetylcholine receptor or one of its peptides would be a treatment for myasthenia gravis (10); p277 variant plus antigen S or one of its peptides for treatment of uveitis (11), and so forth for other autoimmune diseases or inflammatory conditions. The p277 variant itself would be a treatment to inhibit rejection of islet grafts, or of other allografts.
5. Inhibition of chemokine receptor-related conditions. It is known that the HIV virus uses CXCR4 as a co-receptor for infecting cells (12). In other words, CXCR4 is a co-receptor for HIV (12), and inhibition of CXCR4 would inhibit HIV infection. Since p277 down-regulates CXCR4 expression, p277 or its analogues would be useful in providing resistance to HIV infection. Any future discovery of the involvement of CXCR4 or CRC7 in any infection or other disease or pathology would be amenable to treatment with p277 or its analogues.

Preferred embodiments of the invention

According to one aspect of the present invention, the epitope of HSP60 or p277 or an active fragment thereof capable of eliciting activity via the TLR-2, is conjugated to a second segment capable of eliciting a reaction via a T cell receptor (TCR) to form a dual-ligand complex. Thus, the TLR-2 reacting epitope of HSP60 or p277 or an active fragment thereof is combined with molecules or peptides known to be targeted as antigens in other autoimmune diseases, wherein the peptide epitopes are covalently linked to one another directly or through a spacer such as by glycine or alanine residues, or via any other linker as is well known in the art to which the invention pertains. This antigenic fragment provides specificity to specific T cell via the TCR, while the TLR-2 reacting epitope derived from HSP60 or p277 provides the means of

diminishing damaging immune responses and enhancing beneficial immune responses.

- The second segment capable of eliciting a reaction via a TCR used in the
- 5 present invention may be an antigen involved in any one or more of the following autoimmune diseases or disorders: diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, myasthenia gravis, systemic lupus erythematosus (SLE), autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis),
- 10 psoriasis, Sjogren's Syndrome, including keratoconjunctivitis sicca secondary to Sjogren's Syndrome, alopecia areata, allergic responses due to arthropod bite reactions, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema
- 15 nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Graves ophthalmopathy, sarcoidosis,
- 20 primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis.

Examples of known antigens involved in autoimmune diseases include but are not limited to myelin basic protein, MOG or PLP (involved in multiple sclerosis), acetylcholine receptor components (involved in myasthenia gravis), collagen or Mycobacterial hsp peptide (involved in arthritis), and laminin or p53 peptide

25 (involved in systemic lupus erythematosus).

It is to be understood that the compositions and methods of the present invention are amenable for use with these and other known peptide antigens that have been implicated as T cell epitopes involved in autoimmunity. Clearly the present invention is intended to encompass any other such peptide antigens that may in future

30 be disclosed that may be used as the TCR interacting segment of the Dual Effect Ligands according to the principles of the present invention.

In a preferred embodiment, the TLR-2 reacting epitope of HSP60 or p277 or an active fragment thereof, is combined to a myelin basic protein fragment, for the

treatment of multiple sclerosis. In a preferred embodiment, the myelin basic protein peptide having for example the sequence disclosed in U.S. Patent No. 6,489,299, denoted herein as SEQ ID No. 3: Pro Lys Tyr Val Lys Gln Asn Thr Leu Lys Leu Ala Thr (MBP 87-99).

- 5 In another preferred embodiment, the TLR-2 reacting epitope of HSP60 or p277 or an active fragment thereof, is combined to acetylcholine receptor antigen or one of its peptides for the treatment of myasthenia gravis. In a preferred embodiment, the acetylcholine receptor peptides used are the p259 peptide (see Zisman et al., Hum Immunol. 1995 Nov; 44 (3): 121-30 and Brocke et al., Immunology 1990 Apr; 69 (4) : 495-500).
- 10 In another preferred embodiment, the acetylcholine receptor peptides used are fragments which comprise the amino acid residues 61-76 of the hAChR or fragments which comprise the amino acid residues 184-210 of the hAChR.

- In another embodiment, the TLR-2 reacting epitope of HSP60 or p277 or an active fragment thereof, is combined to collagen fragment for the treatment of
- 15 arthritis. In a preferred embodiment, the collagen fragment is for example a collagen type CII peptide 245-270 having the sequence disclosed in U.S. Patent No. 6,423,315 and denoted herein as SEQ ID No. 4: P T G P L G P K G Q T G E L G I A G F K G E Q G P K.

- In another embodiment, the TLR-2 reacting epitope of HSP60 or p277 or an
- 20 active fragment thereof, is combined to laminin fragment for the treatment of systemic lupus erythematosus. In various embodiments of the present invention, peptides derived from the C-terminal or N-terminal of mouse laminin chain may be used.

Amino acid sequences of laminin fragments are disclosed for example in U.S.

Patent No. 6,228,363 and denoted herein as:

- 25 SEQ ID No. 5: RPVRHAQCRVCDGNSTNPRERH;
SEQ ID No. 6: KNLEISRSTEDLLRNSYGVRK;
SEQ ID No. 7: TSLRKALLHAPTGSYSDGQ;
SEQ ID No. 8: KATPMLKMRTSFHGCIK;
SEQ ID No. 9: DGKWHTVKTEYIKRKAF;
- 30 SEQ ID No. 10: KEGYKVRDLNITLFRITTSK; and
SEQ ID No. 11: KQNCCLSSRASFRGCVRNLRLSR.

The antigenic fragments which provides specificity to specific T cell via the TCR peptides may be prepared for example by the F-moc technique (Carpino LA & Han GY (1972) J Org Chem 37 3404), or any other method of peptide synthesis known to those skilled in the art, such as for example by solid phase peptide synthesis.

- 5 These fragments could also be produced by methods well known to one skilled in the art of biotechnology. For example, using a nucleic acid selected from the group including DNA, RNA, or cDNA. The desired fragments may be produced in live cell cultures and harvested.

In one embodiment, the present invention provides a method of treating
10 inflammatory condition or disorder, or a disorder dependent on chemokine receptors, comprising administering to a patient in need thereof an anti-inflammatory effective amount of a pharmaceutical composition comprising as an active ingredient HSP60 or an active fragment thereof. In one embodiment, the active fragment of HSP60 is the p277 peptide. In another embodiment, said fragment is an active fragment of p277.

15

The inflammatory condition or disorder may be selected from chronic inflammatory disorder, acute inflammatory disorder, an autoimmune disease and graft rejection. A disorder dependent on chemokine receptors may be for example HIV infection.

20

The pharmaceutical composition of the invention comprises as an active ingredient HSP60 or an active fragment thereof in an amount sufficient to induce anti-inflammatory effect on T cells. In a preferred embodiment, the active fragment of HSP60 is the p277 fragment.

25

The HSP60 used in the composition of the invention may be human HSP60 or E. Coli HSP60. In a preferred embodiment, the composition of the invention comprises human HSP60, or an active fragment thereof.

30

In another embodiment, the present invention provides a method of treating an inflammatory condition or disorder, or a disorder dependent on chemokine receptors, comprising administering to a patient in need thereof a pharmaceutical composition comprising cells exposed ex vivo to an anti-inflammatory effective amount of HSP60, or an active fragment thereof. In a preferred embodiment, said cell are autologous T cells exposed ex vivo to an effective amount of HSP60 or an active fragment thereof,

sufficient to induce anti-inflammatory effect on T cells. In one embodiment, the active fragment of HSP60 is the p277 peptide. In another embodiment, said fragment is an active fragment of p277.

- 5 The present invention also provides a method of inhibiting the migration of T cells comprising exposing the T cells to soluble HSP60, or an active fragment thereof. The exposure of T cells to HSP60, or an active fragment thereof causes adherence of the T cells to a substrate comprising extracellular matrix components, such as fibronectin, thereby inhibiting the migration of said T cells.

10 Pharmaceutical formulations

- The present invention is also directed to pharmaceutical compositions comprising a pharmaceutically acceptable carrier and at least one anti-inflammatory peptide or peptide derivative of the invention. The pharmaceutical composition will be administered according to known modes of peptide administration, including oral, 15 intravenous, subcutaneous, intraarticular, intramuscular, inhalation, intranasal, intrathecal, intradermal, transdermal or other known routes. The dosage administered will be dependent upon the age, sex, health condition and weight of the recipient, and the nature of the effect desired.

- The peptides of the invention for use in therapy are typically formulated for 20 administration to patients with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. The formulation will depend upon the nature of the peptide and the route of administration but typically they can be formulated for topical, parenteral, intramuscular, intravenous, intraperitoneal, intranasal inhalation, lung inhalation, intradermal or intra-articular administration. The peptide may be used 25 in an injectable form. It may therefore be mixed with any pharmaceutically acceptable vehicle which is suitable for an injectable formulation, preferably for a direct injection at the site to be treated, although it may be administered systemically.

- The pharmaceutically acceptable carrier or diluent may be, for example, sterile isotonic saline solutions, or other isotonic solutions such as phosphate-buffered saline. 30 The peptides of the present invention may be admixed with any suitable binder (s), lubricant (s), suspending agent (s), coating agent (s), solubilizing agent (s). It is also preferred to formulate the peptide in an orally active form.

Tablets or capsules of the peptides may be administered singly or two or more at a time, as appropriate. It is also possible to administer the peptides in sustained release formulations.

Typically, the physician will determine the actual dosage which will be most
5 suitable for an individual patient and it will vary with the age, weight and response of the particular patient.

There can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

Alternatively, the peptides of the invention, can be administered by inhalation or
10 in the form of a suppository or pessary, or they may be applied topically in the form of a lotion, solution, cream, ointment or dusting powder. An alternative means of transdermal administration is by use of a skin patch. For example, they can be incorporated into a cream consisting of an aqueous emulsion of polyethylene glycols or liquid paraffin. They can also be incorporated, at a concentration of between 1 and
15 10% by weight, into an ointment consisting of a white wax or white soft paraffin base together with such stabilizers and preservatives as may be required.

For some applications, preferably the compositions are administered orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions
20 or suspensions containing flavoring or coloring agents. For such oral administration, the peptide may preferably be formed into microcapsules or nanoparticles together with biocompatible polymers such as poly-lactic acid and the like.

The compositions (as well as the peptides alone) can also be injected parenterally, for example intravenously, intramuscularly or subcutaneously. In this
25 case, the compositions will comprise a suitable carrier or diluent. For parenteral administration, the compositions are best used in the form of a sterile aqueous solution, which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood.

For buccal or sublingual administration the compositions may be administered
30 in the form of tablets or lozenges which can be formulated in a conventional manner.

A composition according to the invention can be formulated for parenteral administration by injection or continuous infusion. Compositions for injection can be provided in unit dose form and can take a form such as suspension, solution or emulsion in oil or aqueous carriers and can contain formulating agents, such as suspending, stabilizing and/or dispersing agents. Alternatively, the active constituent can be present in powder form for constitution with a suitable carrier, for example sterile pyrogen-free water, before use. The composition of the invention may be administered directly into a body cavity adjacent to the location of the inflammatory area, such as the intraperitoneal cavity, or injected directly into or adjacent to the inflammatory area.

Peptidomimetics

A peptide mimetic or peptidomimetic, is a molecule that mimics the biological activity of a peptide but is not completely peptidic in nature. Whether completely or partially non-peptide, peptidomimetics according to this invention provide a spatial arrangement of chemical moieties that closely resembles the three-dimensional arrangement of groups in the peptide on which the peptidomimetic is based. As a result of this similar active-site geometry, the peptidomimetic has effects on biological systems which are similar to the biological activity of the peptide.

Without wishing to be bound by theory, the present invention encompasses peptide, peptide analog and peptidomimetic compositions, which are capable of binding to or activating the Th2 receptor of T cells. Said peptide/peptidomimetic compositions are effective in situations where down regulation of the Th1 response is desirable and where up-regulation of the Th2 response is beneficial, including but not limited to autoimmune diseases and graft rejection.

There are clear advantages for using a mimetic of a given peptide rather than the peptide itself, because peptides commonly exhibit two undesirable properties: poor bioavailability and short duration of action. Peptide mimetics offer a route around these two major obstacles, since the molecules concerned are have a long duration of action. Small peptidomimetics of 3-6 amino acids exhibit improved patient compliance since they can be administered orally compared with parenteral administration for peptides or larger peptidomimetics. Furthermore there are problems

associated with stability, storage and immunoreactivity for peptides that are not experienced with peptide mimetics.

One aspect of the present invention provides for a peptidomimetic or a peptide or peptide analog which mimics the structural features of the critical minimal epitope.

5 The design of the peptidomimetics may be based on the three-dimensional structure of the T cell receptors with or in complex with their ligands. Peptidomimetics are small molecules that can bind to proteins by mimicking certain structural aspects of peptides and proteins. They are used extensively in science and medicine as agonists and antagonists of protein and peptide ligands of cellular and
10 other receptors, and as substrates and substrate analogs for enzymes. Some examples are morphine alkaloids (naturally-occurring endorphin analogs), penicillins (semi-synthetic), and HIV protease inhibitors (synthetic). Such compounds have structural features that mimic a peptide or a protein and as such are recognized and bound by other proteins. Binding the peptidomimetic either induces the binding protein to carry
15 out the normal function caused by such binding (agonist) or disrupts such function (antagonist, inhibitor).

A primary goal in the design of peptide mimetics has been to reduce the susceptibility of mimics to cleavage and inactivation by peptidases. In one approach, one or more amide bonds have been replaced in an essentially isosteric manner by a
20 variety of chemical functional groups. In another approach, a variety of uncoded or modified amino acids such as D-amino acids and N-methyl amino acids have been used to modify mammalian peptides. Alternatively, a presumed bioactive conformation has been stabilized by a covalent modification, such as cyclization or by incorporation of γ -lactam or other types of bridges as disclosed for example in US
25 patent 5,811,392. In US Patent 5,552,534, non-peptide compounds are disclosed which mimic or inhibit the chemical and/or biological activity of a variety of peptides. Such compounds can be produced by appending to certain core species, such as the tetrahydropyranyl ring, chemical functional groups which cause the compounds to be at least partially cross-reactive with the peptide. As will be recognized, compounds
30 which mimic or inhibit peptides are to varying degrees cross-reactive therewith. Other techniques for preparing peptidomimetics are disclosed in US Patent 5,550,251 and US Patent 5,288,707, for example. Non-limiting examples of the use of peptidomimetics in the art include inhibitors of protein isoprenyl transferases

(particularly protein farnesyltransferase and geranylgeranyltransferase) and anti-cancer drugs (US patent 5,965,539) inhibitors of p21 ras (US patent 5,910,478) and inhibitors of neurotrophin activity (US patent 6,291,247).

- Having now generally described the invention, the same will be more readily understood through reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention.

EXAMPLES

- 10 Reagents. The following reagents and chemicals were purchased from the sources indicated: RPMI-1640 (Gibco BRL), FCS, HEPES buffer, antibiotics, sodium pyruvate (Kibbutz Beit-Haemek; Israel); fibronectin (FN; Chemicon, Temecula, CA); recombinant human IL-2 (Chiron; Amsterdam, The Netherlands); SDF-1 α , ELC (R&D Systems; Minneapolis, MN). Tissue culture plates were obtained from Becton
- 15 Dickinson Labware (Franklin Lakes, NJ). monoclonal antibodies directed against human β integrins (CD29), and their α chain subunits, α_4 (VLA4), α_5 (VLA5) α_6 (VLA 6) were obtained from Serotec (Oxford, U.K.). Monoclonal antibodies directed against toll-like receptor-2 (TLR-2) or toll-like receptor-4 (TLR-4) were obtained from eBioscience (San-Diego, CA). Monoclonal antibody anti-phosphorylated Pyk2
- 20 (clone py881) was obtained from Biosource (Camarillo, CA), and anti-total Pyk2 (clone N-19) from Santa-Cruz Biotech (Santa-Cruz, CA).

- Human T cells. T cells from human peripheral blood were isolated on Ficoll-Histopaque pre-packed columns and washed. B cells were depleted by adherence to
- 25 nylon wool (Novamed; Jerusalem, Israel) in a humidified atmosphere (45 min, 37°C, humidified atmosphere, humidified incubator, 7% CO₂) as previously described (15). The non-adherent cells were eluted and washed. To remove monocytes, we incubated (2 h, 37°C, 7% CO₂, humidified atmosphere) the cells on tissue culture grade petri dishes. The nonadherent cells were collected and CD3⁺ T cells were isolated by
- 30 negative selection with a mouse anti-human antibody cocktail (Pan T cell kit Miltenyi Biotec; Germany) containing mAb against CD11b, CD16, CD19, CD36, and CD56. The labeled cells were then passed through separation columns (MiniMACS columns, Miltenyi Biotec). In a second round of purification, CD3⁺ T cells were

labeled for negative selection with magnetically coupled mAb against CD45RA⁺ and CD45RO⁺ (Miltenyi Biotec). Unlabeled T cells were subsequently collected and analyzed by FACS for CD45RO⁺ or CD45RA⁺ expression. The purified cells obtained (usually >97% CD45RO⁺ or CD45RA⁺ T cells) were cultured in RPMI

- 5 containing 10% heat-inactivated FCS.

T-cell adhesion assay. Analysis of T-cell adhesion to ECM components was determined as previously described (15, 16). Briefly, flat-bottom microtiter well plates were precoated with FN (10 µg/ml each; Sigma, St. Louis, MO) and the remaining binding sites were blocked with 1% BSA. Next, ⁵¹[Cr]-labeled T cells were resuspended in RPMI medium supplemented with 1% HEPES buffer and 0.1% BSA (adhesion medium), preincubated (30 min, 37°C, 7.5% CO₂ in a humidified atmosphere) with p277, and were then added to the wells. The plates were incubated (30 min, 37°C in a 7.5% CO₂ humidified atmosphere) and then gently washed x3. The adherent cells were lysed (1M NaOH, 0.1% Triton X-100 in H₂O), removed, and counted by a γ-counter (Packard, Downers Grove, IL). The results (±SD) are expressed as the mean percentage of bound T cells from quadruplicate wells.

Chemotaxis and migration assays:

- 20 T cell chemotaxis within ECM-like gels, containing collagen type I, FN, and laminin, was performed and analyzed as described (26, 27). Briefly, purified CD45RA⁺ and CD45RO⁺ T cells were mixed in a drop of ECM-gel (drop I, 150-200 cells within 10 µl of gel). A second drop (drop II, 8 µl of gel) was placed ~2 mm from drop I on the surface of a glass slide within a Lab Tek chamber (Nunc; Denmark). A chemoattractant depot was created by a third drop of the gel (drop III, 3 µl of gel) containing 100 ng/ml SDF-1α or ELC, 1.5 mm from drop II and 3.5 mm from drop I, within the same chamber. The drops were preincubated (30 min, 37°C, 7.5% CO₂ humidified atmosphere) and drop I and drop II were then gently connected with a 29-gauge syringe needle to form a continuous matrix. After an additional 15 min, the chemoattractant depot was connected with drop II to allow the chemoattractive gradient to form. The chambers were immediately placed into humidified, CO₂-filled chambers and applied onto the stage of a microscope that was heated to 37°C. Where indicated, T cells were pre-incubated (1 hr, 37°C in a 7.5% CO₂ humidified
- 30

atmosphere) with different concentrations of HSP60 and the treated T cells were washed and placed into the gel. T cell migration within the ECM-like gels was monitored using an inverted phase-contrast Diaphot Microscope (Nikon, Japan). T cell movement within a selected focal plane of the gel proximal to the slide (between drops I and II, approximately 320 nm) was photographed and recorded on a time-lapse video recorder (AG-6730 S-VHS; Panasonic; Japan) at 25 frame/min. Directionally moving cells were T cells that migrated in the direction of the chemoattractant for a least 15-20 min and during the time period of the assay (60 min). For each time interval, T cell movement over a 10 min period was analyzed.

Purified human CD45RA⁺ and CD45RO⁺ T cells were treated for 18 hr with the indicated concentrations of p277, washed and cell migration was measured using a Transwell apparatus (6.5-mm diameter; Corning, NY) fitted with polycarbonate filters (5 μ m pore size). The filters, which separated the upper and lower chambers, were pre-treated (1 hr, 37°C) with FN (25 μ g/ml). Aliquots (100 μ l) of ⁵¹[Cr]-labeled T cells (2x10⁶/ml of RPMI containing 0.1% BSA, 0.1% L-glutamine, and antibiotics) were added to the upper chambers. The bottom chambers contained 0.6 ml of the same media with or without human SDF-1 α (250 ng/ml). After 3 hr (37°C, 7.5% CO₂-humidified atmosphere), T cell migration through the FN-coated filters was determined by collecting the transmigrated cells from the lower chambers. These cells were centrifuged and resuspended in 100 μ l of distilled water containing 1M NaOH and 0.1% Triton X-100, and counted in a γ -counter. The percentage (\pm SD) of cell migration was calculated as the number (counts) of the migrating cells (residing in the lysates from the lower chamber) out of the total counts (100 μ l aliquot of the starting 2x10⁶ cells/ml).

Western blot analysis of T-cell lysates. Purified human CD45RA⁺ and CD45RO⁺ T cells were incubated in starvation medium (RPMI medium without serum) for a least 24 hr (26). Before the experiments, 5x10⁶ cells per sample were activated with PMA (50 ng/ml) or with different concentrations of HSP60 (15 min, 37°C in a 7.5% CO₂ humidified atmosphere). The reaction was terminated by freezing the plates at -70°C for 10 min. The thawed cells were incubated (60 min, 4°C) in lysis buffer containing EDTA (0.5 mM), NaCl (150 mM), NaF (10 nM), Tris pH 7.5 (25 mM), Triton X-100

(1%), PMSF (200 $\mu\text{g/ml}$), and phosphatase inhibitor cocktail (1%; Sigma), cleared by centrifugation (30 min, 14×10^3 rpm), and the supernatants analyzed for protein content. Sample buffer was then added, and after boiling, the samples were separated on 10% SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes
5 were blocked [TBST buffer containing low-fat milk (5%), Tris pH 7.5 (20 mM), NaCl (135 mM) and Tween 20 (0.1%)], and probed with the following antibodies. The types of antibodies used were anti-phosphorylated Pyk2 (pPyk2) and anti-total Pyk2 (tPyk2): 1.5 and 0.2 $\mu\text{g/ml}$, respectively. Immunoreactive protein bands were visualized using horseradish peroxidase-conjugated secondary Ab and the enhanced
10 ECL system.

Actin polymerization. Human CD45RA⁺ T cells (3×10^6 cells per ml) were preincubated with HSP60 (1 hr, 37°C in a 7.5% CO₂ humidified atmosphere) and treated with 200 ng/ml of SDF-1 α for 15 sec at 37°C and then fixed by the addition of
15 a threefold volume of 3.7% PFA for 10 min at 22°C (26). Next, the cells were extensively washed and the membranes were permeabilized (2 min) in a solution containing HEPES (20 mM), Sucrose (300 mM), NaCl (50 mM), MgCl₂ (3 mM), and TritonX-100 (0.1%). Thereafter, the T cells were stained (30 min) with FITC-phalloidin (2 $\mu\text{g/ml}$), washed with PBS and analyzed by FACScan (Beckton
20 Dickinson; Mountain View, CA) at 525 nm, using Cell Quest Software (26).

Adoptive transfer of delayed-type hypersensitivity (DTH) Assay. Donor female inbred BALB/c mice were sensitized on the shaved abdominal skin with 100 μl of 2% oxazalone (Sigma Chemicals, Inc.) dissolved in acetone/olive oil [4:1 (vol/vol)]
25 applied topically. On day 6, draining peripheral lymph nodes were removed, washed, and a single cell suspension was made. Lymph node cells were counted and incubated (10^7 cells/ml) with 250 ng/ml of HSP60 in RPMI containing 10% heat-inactivated FCS for 18 hr. or with the medium alone (control). DTH sensitivity was elicited in naïve recipient syngeneic mice by i.v. injecting the control or HSP60-treated cells
30 (50×10^6 cells/mouse), followed by immediate challenge with 20 μl of 0.5% oxazalone in acetone/olive oil, 10 μl applied topically to each side of their ears. A constant area of the ear was measured immediately before challenge and 24 hr after challenge with a Mitutoyo engineer's micrometer. The DTH reaction is presented as the increment of

ear swelling after challenge expressed as the DTH index (mean – normal ear) in units of 10^{-2} mm \pm SD.

EXAMPLE 1: Human HSP60 and the p277 fragment induce T-cell adhesion to FN.

We purified T cells from the peripheral blood of healthy human blood donors, fractionated the T cells into CD45RA⁺ (“naïve”) and CD45RO⁺ (“memory”) subpopulations, incubated the T-cells with various concentrations of human HSP60 for various times, and assayed adhesion of the T cells to immobilized ECM components: FN, laminin or collagen. We found that HSP60, which did not affect cell viability, enhanced T-cell adhesion specifically to FN; there was little or no effect on T-cell adherence to laminin or collagen Type I. The adhesion experiments presented here use, therefore, immobilized FN as the ECM substrate.

Figure 1 shows the effects of various concentrations of the p277 fragment of HSP60 on T-cell adhesion to FN, measured as the percent increase in adhesion above background. It can be seen that the non-activated CD45RA⁺ population responded to relatively low concentrations of p277 (0.1-1.0 ng/ml). The non-activated CD45RO⁺ population responded mostly to the higher concentrations (>100 ng/ml) of p277.

Figure 9A shows the effects of various concentrations of human HSP60 on T-cell adhesion to FN, measured as the percent increase in adhesion above background (7 \pm 3% background adhesion). It can be seen that the non-activated CD45RA⁺ population responded to relatively low concentrations of HSP60 (0.1-1.0 ng/ml). Adhesion decreased at concentrations of 10-100 ng/ml, and then increased again at concentrations of 1000-5000 ng/ml. The non-activated CD45RO⁺ population responded mostly to the higher concentrations (>100 ng/ml) of HSP60.

CD45RO⁺ T cells include memory T cells that are responsive to activation by IL-2; we therefore tested whether pre-activation with IL-2 might influence the response of the CD45RO⁺ T cells. Figure 9A shows that activation by IL-2 increased the sensitivity to HSP60 of the CD45RO⁺ T-cell population by 1000 fold. Thus the state of a T cell can influence the way it responds to different concentrations of HSP60; non-activated CD45RO⁺ T cells, like macrophages, respond to HSP60 in the μ g/ml range, but activated CD45RO⁺ T cells, like non-activated CD45RA⁺ T cells,

can respond to HSP60 by enhanced adhesion to the immobilized ECM glycoprotein in the ng/ml range.

Figure 9B shows the time course of T cell adhesion to immobilized FN induced by pre-incubation with HSP60 for 0.5 to 48 hours. It can be seen that HSP60 exerted a rapid adhesive effect on human T cells; 1 hour of contact with an optimal concentration of HSP60, 0.1 ng/ml for CD45RA⁺ cells and 1 µg/ml for non-activated CD45RO⁺ cells, induced peak levels of T cell adhesion to FN (120% and 140% increase, respectively). Therefore, we used the 1 hour incubation in the experiments described here.

EXAMPLE 2: *E. coli* HSP60 (GroEL) induces T-cell adhesion to FN

Purified human T cells were radiolabeled, pretreated (30 min, tissue culture conditions) with different concentrations of GroEL (A) or LPS (B), and seeded onto FN-coated microtiter wells. Percent adhesion was determined 30 min later as in

Figure 9. Mean±SD of five experiments is shown.

Figure 10A shows the response to low concentrations of the HSP60 molecule of *E. coli* (GroEL). It can be seen that GroEL also enhanced the adhesion of the CD45RA⁺ population, but to a lesser degree than the adhesion induced by human HSP60 (compare Figures 9 and 10A). However, not all species of HSP60 are able to activate adhesion of CD45RA⁺ T cells at ng/ml concentrations; the HSP60 molecule of *Mycobacterium tuberculosis* (HSP65) was not active in this assay (not shown).

Although the level of LPS in the batch of HSP60 we used was low (LPS <0.001 EU/ml), we were concerned that even minimal residual LPS contamination might have affected T cell adhesion. Accordingly, we tested the direct effects of LPS. Figure 10B shows the effect on T-cell adhesion to FN of treating non-activated CD45RA⁺ or CD45RO⁺ T cells with various concentrations of LPS. The undulating form of the LPS dose-response curve was similar to that observed in response to human HSP60. Note, however, that a 1000-fold greater concentration of LPS was needed and the maximal adhesion response was still less than that induced by human HSP60 (compare Figures 9A and 10B). Thus, LPS contamination of the HSP60 could not account for the induction of T cell adhesion. Moreover, the sensitivity of the innate responses to LPS and to HSP60 seems to be reversed in T cells and in macrophages:

CD45RA⁺ T cells are more responsive to HSP60 and macrophages are more responsive to LPS.

EXAMPLE 3: HSP60- and p277-induced T-cell adhesion depend on $\beta 1$ integrins

Information about molecular mechanisms involved in the T-cell response to HSP60 is shown in Figure 11. Figure 11A shows that HSP60-induced adhesion was significantly inhibited by monoclonal antibodies to VLA4, VLA5 (the FN-specific $\alpha 4$ and $\alpha 5$ chains of $\beta 1$ integrins), or CD29 (the common $\beta 1$ integrin chain); in contrast, control monoclonal antibodies to VLA2 ($\alpha 2\beta 1$), VLA3 ($\alpha 3\beta 1$), or CD44 did not affect HSP60-induced adhesion. It is noteworthy that 1 hour pretreatment of the two T cell subpopulations did not affect the expression levels of FN specific $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins (data not shown). Thus, existing FN-specific $\beta 1$ integrins on T cells would appear to be activated in HSP60-induced adhesion. This adhesion appears to involve PI-3 kinase and PKC signaling because it was inhibited (Figure 11B) by the compounds wortmanin and GF109203X (GF), respectively, but not by pertussis toxin (PTX), which inhibits specifically G protein-coupled signaling.

The cytoplasmic tyrosine kinase 2 (Pyk-2) is a member of the focal adhesion kinase (FAK) family of molecules which are phosphorylated upon PKC activation. Tyrosine phosphorylation of Pyk2 (and related molecules) increases the activity of this kinase and links $\beta 1$ integrins to multiple signaling pathways regulating adhesion and migration processes in T lymphocytes. Such intracellular processes are also involved in the regulation of mitogen-activated protein (MAP) kinase (such as ERK-2) and Jun-NH₂ kinase pathways. Figure 11C shows that a short treatment (15 min) of non-activated CD45RA⁺ T cells with HSP60 (0.01-100 ng/ml) induced the phosphorylation of Pyk-2. Note that similar to T-cell adhesion to FN (see Figure 9A), Pyk-2 phosphorylation showed a bell-shaped dose-response to rising concentrations of HSP60, reaching peak phosphorylation at 1 ng/ml HSP60; treatment of non-activated CD45RO⁺ T cells with ng/ml concentrations of HSP60 did not induce Pyk-2 phosphorylation (not shown). Thus, Pyk-2 phosphorylation accompanies the response of T cells to effective concentrations of HSP60.

Similar to HSP60, the p277 fragment of HSP60 induced adhesion which was significantly inhibited by monoclonal antibodies to VLA4 and VLA5 (Figure 2).

EXAMPLE 4: TLR-2 is involved in T-cell adhesion induced by HSP60 and p277.

The innate TLR-4 receptor, which is involved in LPS signaling, is required for the activation of macrophages and other cells by HSP60. Our finding that T cells were markedly more sensitive to HSP60 than they were to LPS (Figure 9), and our inability to find TLR-4 on CD45RO⁺ or CD45RA⁺ T cells by Western blot (not shown) suggested that an innate receptor other than TLR-4 might be involved in T-cell adhesion induced by soluble HSP60. We therefore tested the effect of HSP60 on the induction of T-cell adhesion in mice bearing a mutated, non-functional TLR-4 molecule. Figure 12A shows that the thymocytes of the wild type C3HeB/FeJ and TLR-4-mutated C3H/HeJ strains were both activated to adhere to FN by 0.1-1.0 ng/ml HSP60. Thus, mouse T cells too can respond to HSP60 by enhanced adhesion to FN, and this response does not require an intact TLR-4 receptor.

It has been reported that T cells express the innate receptor TLR-2, and that TLR-2 is required for a response to LPS; T-cell TLR-2 mRNA was found to be up-regulated in response to IL-2, PMA, and monoclonal anti-CD3 Ab, and was inhibited by inhibitors of kinases. Using Western blot analysis and monoclonal antibody reagents, we also found that TLR-2, but not TLR-4, is expressed in naïve and memory T-cells (data not shown). These observations suggested the possibility that activation of human T cells by HSP60 might involve TLR-2. Figure 12B shows that the monoclonal antibody to TLR-2 could indeed inhibit over 90% of the HSP60-induced T cell adhesion to FN. This inhibition was specific; the anti-TLR-2 antibody did not block T-cell adhesion induced by SDF-1 α (also designated CXCL12) or IL-2 (Figure 12C). In addition, a control monoclonal antibody to TLR-4, of the same isotype as the effective anti-TLR-2 monoclonal antibody, did not affect HSP60-induced adhesion (Figure 12B). Moreover, Western blot analysis of lysates of human T cells and monocytes revealed that the monoclonal anti-TLR-2 antibody recognized identical proteins in both cell types (data not shown). These findings are consistent with the conclusion that TLR-2, rather than TLR-4, is involved in the innate T-cell response to HSP60.

Similar to the effect of TLR-2 antibodies on HSP60-induced adhesion to FN, Figure 3 shows that the monoclonal antibody to TLR-2 could inhibit over 50% of the p277-induced T cell adhesion to FN. In addition, a control monoclonal antibody to TLR-4, of the same isotype as the effective anti-TLR2 monoclonal antibody, did not affect p277-induced adhesion.

EXAMPLE 5: HSP60 inhibits T-cell chemotaxis and actin polymerization.

The ability of T cells to navigate through the ECM depends on the combined signals mediated by pro-adhesive mediators like cytokines and chemoattractants, associated with ECM glycoproteins. Using a 3-dimensional, ECM-like, gel system designed to follow, in real time, the migration of individual leukocytes along chemotactic gradients *in vitro*, we have found that IL-2, RANTES, or SDF-1 α gradients can activate human CD4⁺ T-cell chemotaxis along the ECM. We found that HSP60 alone, presented into the chemokine zone (drop III) at the beginning of the assay, did not activate CD45RA⁺ T-cell chemotaxis (not shown), although it did induce adhesion (see Figure 9). We then tested the effect of HSP60 pre-treatment on T-cell chemotaxis induced by the chemokine SDF-1 α . The untreated or HSP60-treated naïve T cells were embedded in the ECM gels, onto which a chemokine depot (SDF-1 α or ELC, Figures 13A and B, respectively) was previously introduced, and the directional migration of the cells was monitored over time as previously described. Figure 13A shows that about 30% ($P < 0.05$) of untreated CD45RA⁺ T-cells moved toward the SDF-1 α source; there was no directional movement in the absence of SDF-1 α . Pre-treatment of the human T cells with a concentration of 0.1 ng/ml of HSP60, however, inhibited about 2/3 of the T cells migrating toward SDF-1 α ($P < 0.01$). A higher concentration of HSP60, 10 ng/ml, was less effective (about 33% inhibition). Thus, a concentration of HSP60 that induces adhesion also inhibits T-cell migration towards SDF-1 α . The same results were obtained when p277 fragment was used instead of HSP60 with SDF-1 α as a chemoattractant (Figure 4).

T cells have also been shown to respond to the chemokine ELC (also designated CCL19), a prototypic lymph node chemokine that attracts migrating dendritic cells and naïve T cells to T cell areas within the lymph node. Figure 13B shows that HSP60, at 0.1 ng/ml, also inhibited significantly (60% inhibition at $t = 30$ minutes) the migration of T cells towards ELC. Thus, HSP60 induced inhibition of ECM chemotaxis to both SDF-1 α -and ELC.

T cell adhesion to and migration through immobilized ECM ligands requires a dynamic and rapid reorganization of cortical actin, actin polymerization, and

subsequent cellular polarization. Moreover, cytoskeletal-dependent changes in cell shape and motility cause a redistribution of accessory adhesion receptors to specific sites on the cell surface, and thereby, increase leukocyte motility. Therefore, we examined the effects of HSP60 on the rapid induction of actin polymerization. Naïve (CD45RA⁺) T cells were left intact or treated with HSP60 (1 hr, 1 ng/ml), and then activated by SDF-1 α (100 ng/ml) for 15 seconds. The proportion of redistributed F-actin in untreated control T cells, determined by the intracellular staining of the cells with an antibody specific for actin, was considered to be 100%. Figure 13C shows that, similar to its inhibition of migration through ECM, HSP60 inhibited actin polymerization triggered by SDF-1 α . Moreover, this inhibitory effect of HSP60 was itself inhibited by anti-TLR-2 monoclonal antibody, but less so by the isotype-matched anti-TLR-4 monoclonal antibody. Hence, the effect of HSP60 on the rearrangement of the cytoskeleton, and its associated signaling molecules, which are implicated in β 1 integrin-mediated T cell adhesion, also involves TLR-2 signaling.

15

EXAMPLE 6: HSP60 and the p277 fragment inhibit CXCR4 and CCR7 chemokine receptor expression.

The inhibition of T-cell migration to SDF-1 α (Figure 13) raised the question of the effect of HSP60 on the expression of the chemokine receptor CXCR4, which is involved in T-cell chemotaxis towards SDF-1 α . Accordingly, we incubated human T cells with various concentrations of HSP60 and assayed the expression of the chemokine receptors CXCR4 and CCR7, the ELC-specific receptor, at various times. We found that 18 hr of incubation sufficed to reduce specifically the expression of these receptors. Figures 14A and 14B show that 0.1 ng/ml of HSP60 down-regulated the expression of CXCR4 and CCR7, respectively, by 40-50% in CD5RA⁺ T cells; inhibition of CXCR4 in CD45RO⁺ T cells required higher concentrations of HSP60 (10-1000 ng/ml; Figure 14A). Similar treatment and incubations of HSP60 with either CD45RO⁺ or CD45RA⁺ T cells did not change the expression of β 1 integrins and LFA-1 (data not shown). Figure 14C shows that this down regulation of expression of CXCR4 by HSP60 was blocked by mAb anti-TLR-2, but not by anti-TLR-4. The inhibitor of PKC signaling, GF109203X, also inhibited the effect of HSP60 on CXCR4 expression. Thus, the regulatory effect of HSP60 on CXCR4 expression, like the effect of HSP60 on T-cell adhesion and SDF-1 α -induced actin polymerization, seems to require TLR-2 and PKC signaling. The inhibition of CCR4 and CCR7

30

expression following incubation with HSP60 was also evident when the p277 fragment was used instead of the complete HSP60 molecule (Figures 5 and 6).

EXAMPLE 7: HSP60 inhibits Transwell T cell chemotaxis

- 5 To test whether down regulation of CXCR4 expression might be associated with inhibition of T cell migration, we used a Transwell device, designed to monitor the output amount of migration of cells moving on soluble chemotactic gradients. CD5RA⁺ and CD45RO⁺ human T cells were pre-treated for 18 hr with different amounts of HSP60, washed, radioactively labeled, and placed in the upper chambers
10 of the Transwells. The polycarbonate filters separating the two compartments were pre-coated with FN, and the chemokine SDF-1 α was placed in the lower wells at the beginning of the assay. Figure 15 shows that pre-incubating the T cells with appropriate concentrations of HSP60 for 18 hr significantly inhibited migration to the lower well in the chamber: 0.1 ng/ml for CD45RA⁺ and 10-100 ng/ml for CD45RO⁺
15 T cells.

EXAMPLE 8: HSP60 inhibits mouse T cell migration in vitro and an adoptive DTH reaction in vivo.

- The DTH reaction is mediated by antigen-specific T cells that migrate to
20 antigen deposited in the skin and trigger inflammation. We used an adoptive transfer DTH reaction in BALB/c mice to test whether HSP60 treatment of the donor cells *in vitro* might affect the ability of the T cells to trigger a reaction in the recipients.

- First, we analyzed the effect of HSP60 pretreatment (18 hr) on the ability of mouse cells to migrate towards SDF-1 α . Figure 16A shows that untreated lymph node
25 T cells obtained from naïve BALB/c mice migrated in the Transwell apparatus towards human SDF-1 α . However, pretreatment of these T cells with different amounts of HSP60 (18 hr) resulted in inhibition of migration of the cells; the inhibitory effect at 250 ng/ml was 60%.

- Next, BALB/c mice were sensitized to the skin allergen Oxazolone, and 6
30 days later the draining lymph node cells were removed and cell suspensions were incubated for 18 hr with culture medium containing 250 ng/ml HSP60 or without HSP60 (control). The cells were then collected, washed free of the culture medium and injected intravenously (5×10^7 cells per mouse) into syngeneic recipient mice. The mice were then challenged by application to an ear of 3% Oxazolone. Ear thickness

- was measured 24 hr later to quantify the degree of inflammation. Figure 16B shows that the mice injected with the HSP60-treated donor cells had a 60% decrease in ear thickness compared to the control mice that received donor cells incubated for 18 hr with medium without HSP60. Thus, a T-cell mediated immune reaction in mice can be inhibited by pre-treatment with HSP60 *in vitro* at a concentration that affects memory T cells.

- To examine the HSP60 inhibition of adoptive DTH reaction *in vivo*, a 2% Oxazolone solution was applied to the peritoneal surface of female BALB/c mice on day 0. DTH was induced on day 6 by applying a 0.5% oxazolone solution to the ear. Mice were treated intravenously on day 6 (one injection immediately prior to challenge). % inhibition was calculated according to the formula $[1 - (\Delta \text{ ear thickness in treated} / \Delta \text{ in untreated})]$. As shown in Figure 17, as little as 1 ng of Hsp significantly inhibited delayed type hypersensitivity in these mice.

EXAMPLE 9: p277 inhibits Oxazolone-induced immune reaction *in vivo*.

- The effect of p277 *in vivo* by inhibiting a delayed hypersensitivity reaction was examined. The intravenous injection of 0.01 ng given twice can inhibit by 40% a delayed hypersensitivity reaction elicited to the skin sensitizer Oxazolone. Treatment with an injection of 200 μ g of dexamethasone (the gold standard anti-inflammatory drug) inhibited the reaction by 75% (Figure 7).

Figure 8 shows the effect of p277 delivered by constant infusion of 100 ng per hour over 7 days. Here, p277 inhibited the reaction by 30%; dexamethasone treatment inhibited the reaction by 35%.

EXAMPLE 10: HSP60 treatment down-regulates the secretion of the pro-inflammatory cytokine IFN- γ by T cells activated by a variety of stimuli.

- Human T cells were purified and maintained in culture (RPMI, 10% CCS, 1% Pyr, 1% Glu, 1% antibiotics, 7.5% CO₂, humidified atmosphere) for 15 hr before transfer to RPMI, 0.1% BSA, 1% antibiotics) and treated (1 hr, 37°C) with various concentrations of HSP60. After washing, the cells were activated or not (2 hr, 37°C) by incubation with SDF-1 α (100 ng/ml), RANTES(100 ng/ml), or IL-2 (100 ng/ml). The cells were then washed, resuspended in RPMI without CCS containing 0.1%

- BSA (3×10^6 cells per ml) and re-plated on immobilized anti-CD3 mAb (500 ng/ml), pre-coated (over night, 4°C) to 24-well plates (non-tissue culture grade) in order to stimulate the T cells to secrete cytokines. Supernatants were collected after 24 hr and their content of IFN- γ was determined by ELISA using anti-IFN γ (Pharmingen; San Diego, CA) according to the manufacturer's instructions.

- IFN γ is a major Th1-type pro-inflammatory cytokine, and its production by autoimmune T cells has been implicated in a variety of autoimmune diseases and in graft rejection. The effect of HSP60 on the release of IFN γ by activated T cells was examined. As illustrated in Figure 18, HSP60 inhibits IFN- γ secretion by T cells activated by mitogenic anti-CD3. HSP60 also inhibits IFN- γ secretion by T cells activated by chemokine SDF-1 α and mitogenic anti-CD3 (Figure 19), by chemokine RANTES and mitogenic anti-CD3 (Figure 20) and by cytokine IL-2 and mitogenic anti-CD3 (Figure 21). Therefore, it is significant that innate activation of T cells by HSP60 or p277 (via the TLR-2 pathway, as we have shown here) could down-regulate the production of IFN γ . This indicates that HSP60, and any other ligands that operate via TLR-2, will be useful in down-regulating inflammation in any condition where a pro-inflammatory reaction is detrimental.
- It will be appreciated by a person skilled in the art that the present invention is not limited by what has been particularly shown and described hereinabove. Rather, the scope of the invention is defined by the claims that follow.

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CLAIMS

1. A peptide or peptidomimetic comprising a minimal epitope of p277,
the minimal epitope of p277 consisting essentially of an epitope that
retains the ability to elicit anti-inflammatory activity via the Th2
receptor on T cells, without activating the TCR of these cells, other
than known fragments and variants of HSP60 .
2. A dual effect ligand, comprising a peptide, a peptide analog or a
peptidomimetic having as a first segment a Th2-reacting epitope of
Hsp60 and as a second segment a specific peptide capable of eliciting a
reaction via a T cell receptor (TCR).
3. The dual effect ligand of claim 2 wherein the second segment is an
antigenic epitope that is relevant to an inflammatory condition or
autoimmune disease.
4. The dual effect ligand of claim 2 wherein the second segment is an
antigenic epitope that is specific for a cell mediated autoimmune
disease.
5. The dual effect ligand of claim 3 wherein the second segment is an
antigenic epitope specific for a disease selected from: diabetes
mellitus; arthritis including rheumatoid arthritis, juvenile rheumatoid
arthritis, osteoarthritis, psoriatic arthritis; multiple sclerosis;
myasthenia gravis; systemic lupus erythematosus; autoimmune
thyroiditis; dermatitis including atopic dermatitis and eczematous
dermatitis; psoriasis; Sjogren's Syndrome, including
keratoconjunctivitis sicca secondary to Sjogren's Syndrome; alopecia
areata; allergic responses due to arthropod bite reactions; Crohn's
disease; aphthous ulcer, iritis; conjunctivitis; keratoconjunctivitis;
ulcerative colitis; asthma; allergic asthma; cutaneous lupus
erythematosus; scleroderma; vaginitis; proctitis; drug eruptions;
leprosy reversal reactions; erythema nodosum leprosum; autoimmune

- uveitis; allergic encephalomyelitis; acute necrotizing hemorrhagic encephalopathy; idiopathic bilateral progressive sensorineural hearing loss; aplastic anemia; pure red cell anemia; idiopathic thrombocytopenia; polychondritis; Wegener's granulomatosis; chronic active hepatitis, Stevens-Johnson syndrome; idiopathic sprue; lichen planus; Graves ophthalmopathy; sarcoidosis; primary biliary cirrhosis, uveitis posterior; and interstitial lung fibrosis.
- 5
6. The dual effect ligand of claim 3 wherein the second segment is an antigenic epitope selected from SEQ ID Nos: 3-11.
- 10
7. The dual effect ligand of claim 3 wherein the antigenic epitope comprising the second segment binds specifically to the TCR, while the Thr2-reacting epitope derived from p277 provides means of diminishing damaging immune responses and enhancing beneficial immune responses.
- 15
8. The dual effect ligand of claim 2 wherein the Thr2-reacting epitope of Hsp60 is a fragment of p277 consisting essentially of an epitope that retains the ability to elicit anti-inflammatory activity via the Thr2 receptor on T cells, without activating the TCR of these cells.
- 20
9. A pharmaceutical composition comprising the peptide or peptide analog according to any one of claims 1-8.
- 25
10. The pharmaceutical composition of claim 9 further comprising a pharmaceutically acceptable carrier, excipient or diluent.
- 30
11. The pharmaceutical composition of claim 10 in a form suitable for administration by parenteral injection.
12. The pharmaceutical composition of claim 11 in a form suitable for oral administration or inhalation.

- 5 13. Use of a peptide comprising a p277 minimal epitope, an analog thereof or a peptidomimetic according to any one of claims 1-8 in preparation of a medicament for treating a condition selected from the group consisting of an autoimmune disease, an inflammatory condition and graft rejection.
- 10 14. Use of a peptide comprising a p277 minimal epitope, an analog thereof or a peptidomimetic according to any one of claims 1-8 in preparation of a medicament for treating a condition dependent on at least one type of chemokine receptor.
- 15 15. Use according to claim 14 wherein the condition dependent on chemokine receptors is HIV infection.
- 20 16. Use of a p277 minimal epitope, an analog thereof or a peptidomimetic as a first segment in a dual effect ligand conjugated to a second segment comprising at least one other antigen or peptide to treat autoimmune diseases, inflammatory conditions, or graft rejection.
- 25 17. Use according to claim 16 wherein the two segments are covalently conjugated.
- 30 18. Use according to claim 16 wherein the two segments are joined by a linker, selected from the group consisting of a direct peptide bond, a peptide bridge, and a non-peptidic linkage.
19. Use of a peptide, a peptide analog or a peptidomimetic according to any one of claims 1-8 for diagnosis or monitoring the progression of a disease or disorder selected from the group consisting of an autoimmune disease, an inflammatory disease or graft rejection.
20. A method of characterizing an active fragment or analog of HSP60 or of peptide p277 effective in activating innate Tlr2 receptors of T cells,

comprising determining the ability of the fragment or analog to activate T-cell adhesion to the extra-cellular matrix protein fibronectin and to inhibit T-cell chemotaxis towards chemokines.

- 5 21. The method of claim 20 further comprising determining the ability of the analog or active fragment to down-regulate the expression of at least one type of chemokine receptor.
- 10 22. The method of claim 21 wherein the analog or active fragment down regulates the expression of CXCR4.
23. The method of claim 20 further comprising determining the ability of the analog or active fragment to inhibit secretion of IFN γ .
- 15 24. A method of treating a disease selected from an autoimmune disease, an inflammatory condition and graft rejection comprising administering to an individual in need thereof a therapeutically effective amount of a peptide according to any one of claims 1 through 8.
- 20 25. A method of treating a condition dependent on at least one type of chemokine receptor comprising administering to an individual in need thereof a therapeutically effective amount of a p277 minimal epitope, an analog thereof or a dual effect ligand according to any one of claims 1-8.
- 25 26. The method according to claim 25 wherein the condition dependent on chemokine receptors is HIV infection.
- 30 27. A method of treating autoimmune diseases, inflammatory conditions, or graft rejection comprising administering to an individual in need thereof a therapeutically effective amount of a dual effect ligand comprising a p277 minimal epitope, an analog thereof or a

peptidomimetic as a first segment conjugated to a second segment comprising at least one other antigen or peptide.

5 28. The method according to claim 27 wherein the two segments are covalently conjugated.

29. The method according to claim 27 wherein the two segments are joined by a linker, selected from the group consisting of a direct peptide bond, a peptide bridge, and a non-peptidic linkage.

10

30. A method for diagnosing or monitoring the progression of a disease or disorder selected from the group consisting of an autoimmune disease, an inflammatory disease and graft rejection comprising exposing cells from the individual to a peptide, a peptide analog or a peptidomimetic according to any one of claims 1-8.

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31. The method of claim 30 wherein the diagnosis or monitoring is performed in vitro or ex vivo.

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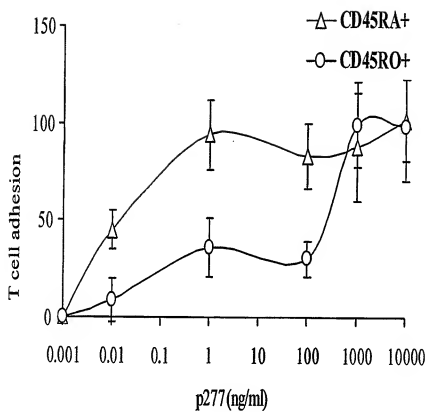


FIG. 1

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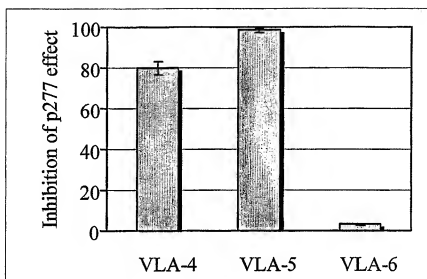


FIG. 2

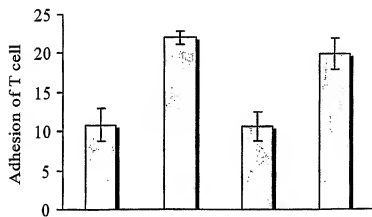


FIG. 3

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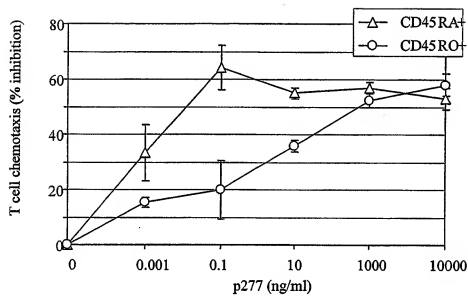


FIG. 4

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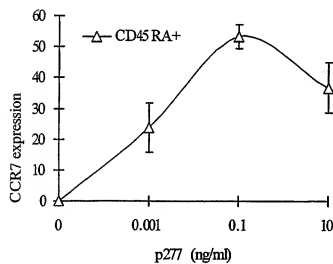
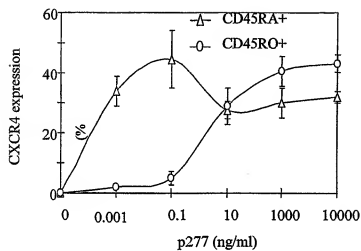


FIG. 5

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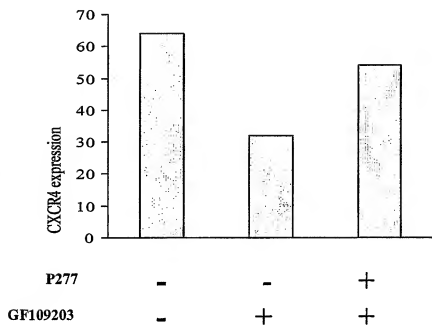


FIG. 6

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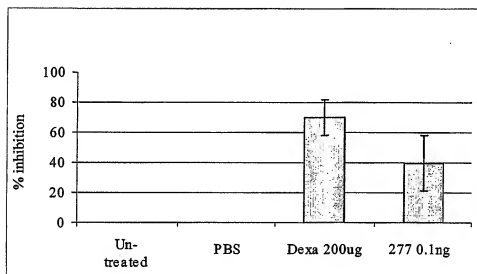


FIG. 7

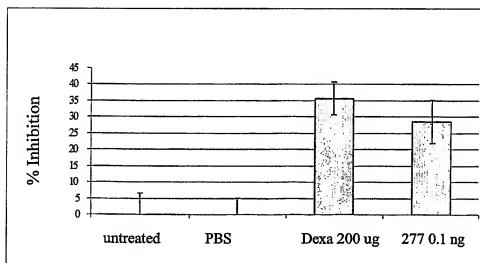


FIG . 8

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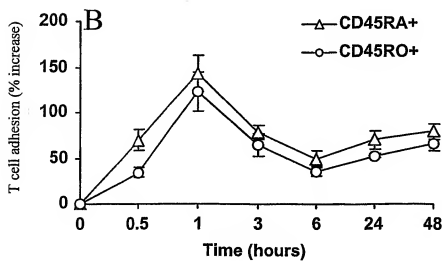
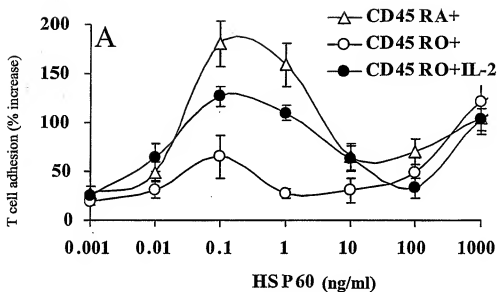


FIG. 9

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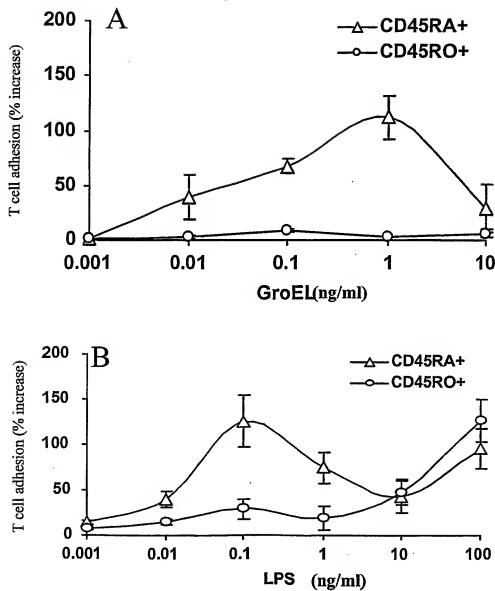


FIG. 10

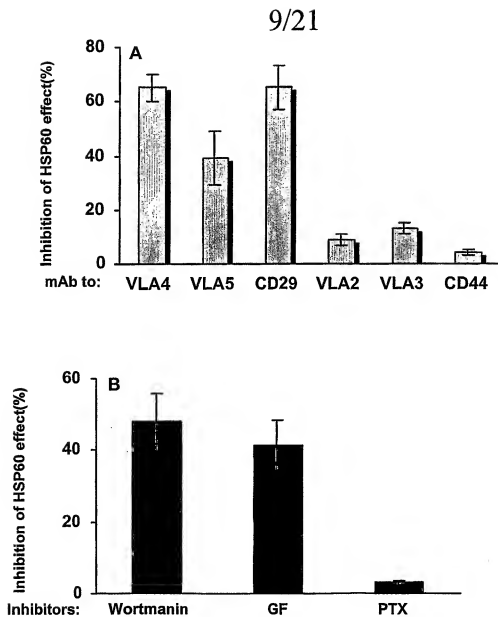


FIG 11 A-B

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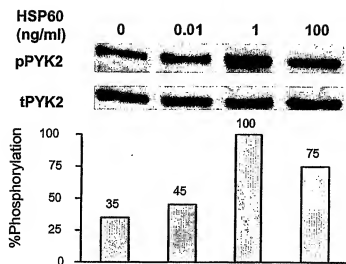


FIG. 11C

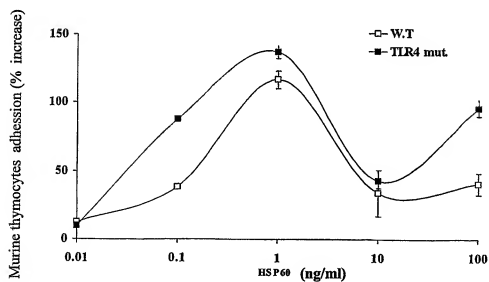


FIG. 12A

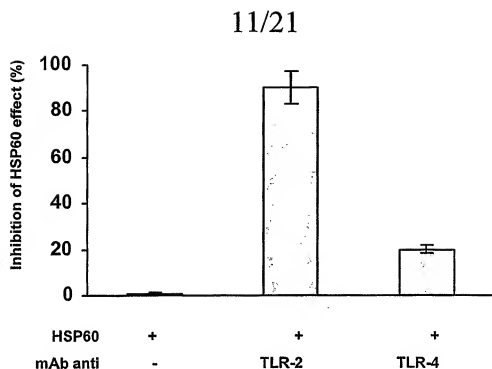


FIG. 12B

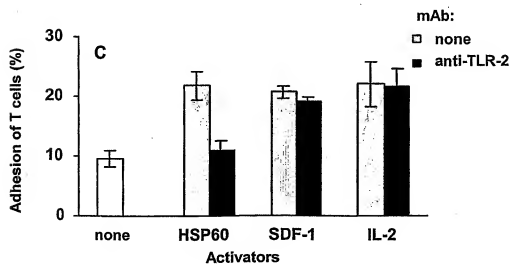


FIG. 12C

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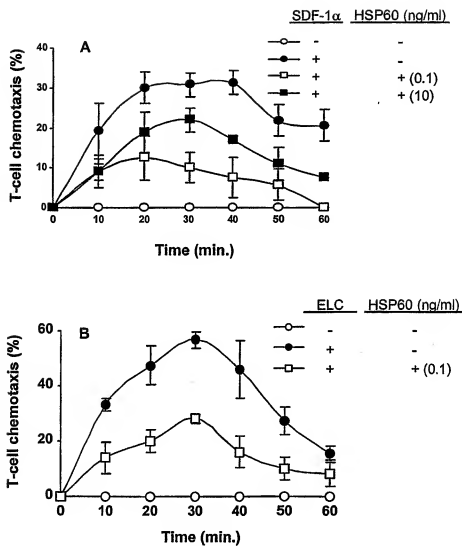


FIG. 13A-B

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C

Modulators of actin polymerization

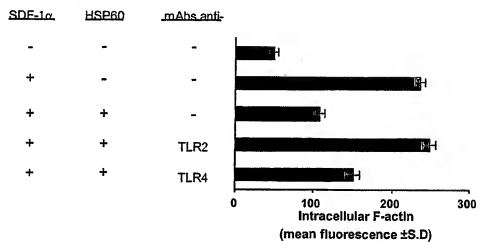


FIG. 13C

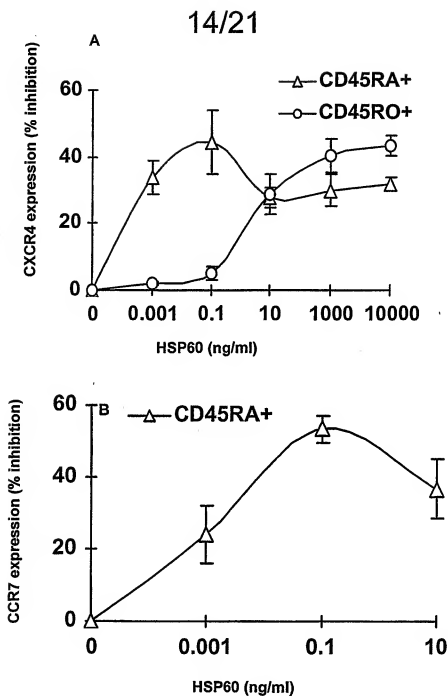


FIG. 14 A-B

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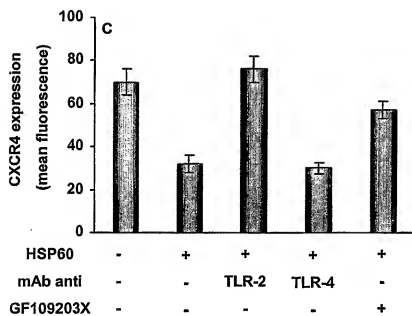


FIG. 14C

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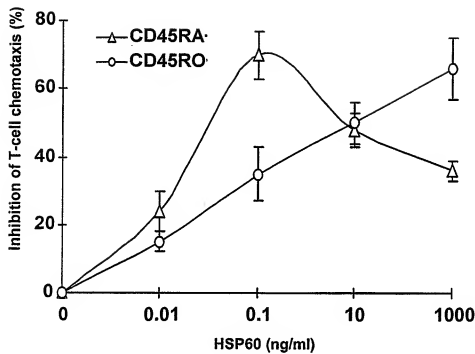


FIG. 15

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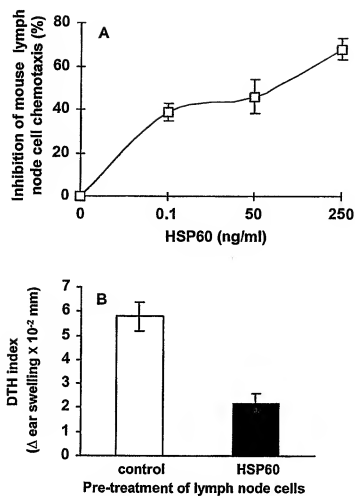


FIG. 16

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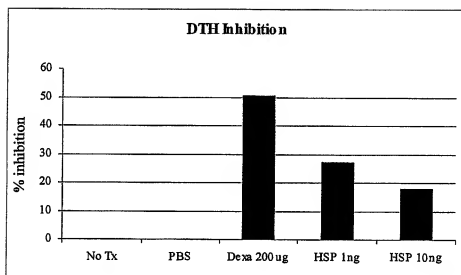


FIG. 17

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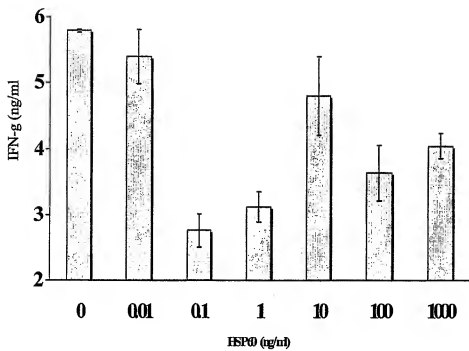


FIG. 18

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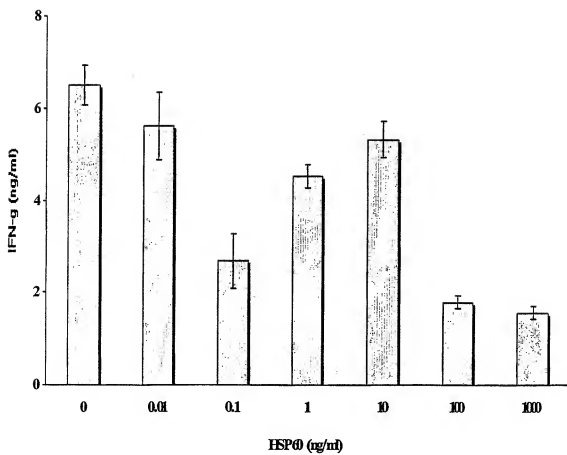


FIG. 19

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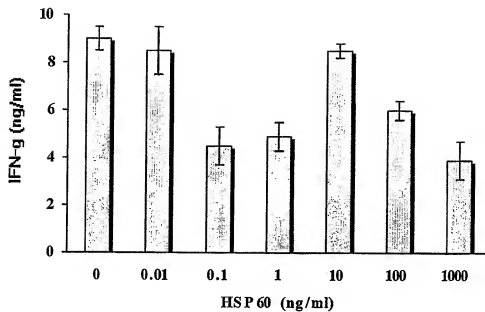


FIG. 20

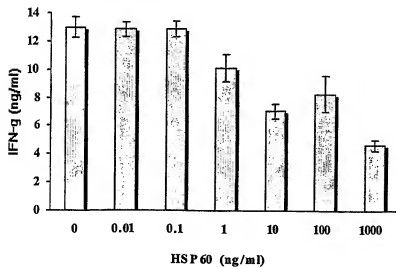


FIG. 21

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C07K14/47 C12N15/62 A61K38/17 A61P37/06 A61P29/00 A61P31/18 G01N33/68		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 C07K A61K C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) BIOSIS, EPO-Internal, WPI Data, PAJ, SEQUENCE SEARCH, EMBASE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 993 803 A (COHEN IRUN R ET AL) 30 November 1999 (1999-11-30) cited in the application the whole document	1-14, 16-19, 24,25, 27-31
X	WO 97 01959 A (ABULAFIA RIVKA ;COHEN IRUN R (IL); ELIAS DANA (IL); YEDA RES & DEV) 23 January 1997 (1997-01-23) cited in the application figure 2; table 2	1-14, 16-19, 24,25, 27-31
X	WO 01 43691 A (KOLB HUBERT ;ELIAS DANA (IL); PEPTOR LTD (IL)) 21 June 2001 (2001-06-21) page 21, line 10 - line 12; example 4; table 2 <div style="text-align: center;">-/-</div>	1-14, 16-19, 24,25, 27-31
<div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. </div>		
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 50%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"S" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search <div style="text-align: center;">18 June 2003</div>		Date of mailing of the international search report <div style="text-align: center;">02/07/2003</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlekan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer <div style="text-align: center;">Madrugá, J</div>

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 180 103 B1 (FRIDKIN MATITYAHU ET AL) 30 January 2001 (2001-01-30) cited in the application claims; examples ---	1-14, 16-19, 24,25, 27-31
X	WO 97 02052 A (COHEN IRUN R ;ELIAS DANA (IL); YEDA RES & DEV (IL)) 23 January 1997 (1997-01-23) the whole document ---	1-14, 16-19, 24,25, 27-31
X	ABLAMUNITS V ET AL: "ISLET T CELLS SECRETING IFN-GAMMA IN NOD MOUSE DIABETES: ARREST BY P277 PEPTIDE TREATMENT" JOURNAL OF AUTOIMMUNITY, LONDON, GB, vol. 11, no. 1, February 1998 (1998-02), pages 73-81, XP000881732 ISSN: 0896-8411 the whole document ---	1-14, 16-19, 24,25, 27-31
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G.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>VABULAS RAMUNAS M ET AL: "Endocytosed HSP60s use Toll-like receptor 2 (TLR2) and TLR4 to activate the Toll/interleukin-1 receptor signaling pathway in innate immune cells." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 276, no. 33, 17 August 2001 (2001-08-17), pages 31332-31339, XP002242849 ISSN: 0021-9258 the whole document</p>	1-31
A	<p>VABULAS R M ET AL: "Heat shock proteins as ligands of Toll-like receptors." CURRENT TOPICS IN MICROBIOLOGY AND IMMUNOLOGY, vol. 270, 2002, pages 169-184, XP008017649 family members and their ligands. 2002 Springer-Verlag New York Inc.;Springer-Verlag GmbH & Co. KG 175 Fifth Avenue, New York, NY, 10010-7858, USA; Heidelberger Platz 3, D-14197, Berlin, Germany ISBN: 3-540-43560-3 the whole document</p>	1-31
X	<p>WO 94 03208 A (FRIDKIN MATITYAHU ;KONEN WAISMAN STEPHANIE (IL); COHEN IRUN R (IL)) 17 February 1994 (1994-02-17) claims</p>	2,3, 7-19, 24-31

INTERNATIONAL SEARCH REPORT

PC1/IL 03/00132

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/SA/ 210

Continuation of Box I.1

Although claims 16-18, 24-29 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Although claims 19 and 30 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

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